Responses of juvenile sea bass, Dicentrarchus labrax, exposed to acute concentrations of crude oil, as assessed by molecular and physiological biomarkers.

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Abstract

In the present study, juvenile sea bass were exposed for 48 and 96 h to an Arabian light crude oil and their responses were assessed at the molecular and physiological levels. The aim of the study was therefore to assess i) the short term effects of crude oil exposure by the measurement of several molecular biomarkers, ii) the consequences of this short term exposure on fish health by using growth and condition indices measured after a decontamination period of 28 and 26 days in seawater. Hydrocarbon petroleum concentrations were followed during the 96 h experiments and an increase of PAH concentrations were found in fish following both exposure time. An EROD induction was observed after 48 h of exposure, while a significant decrease in the sea bass specific growth rate in length and for the RNA:DNA ratio was observed 28 days after that exposure ceased. The EROD induction doubled after the 96 h exposure, and a significant increase in GST activities was observed. A significant decrease in the specific growth rates, the otolith recent growth, the RNA:DNA ratio and the Fulton's K condition index were then observed in sea bass 26 days after the 96 h exposure to mechanically dispersed crude oil compared to the control. The present study shows the usefulness of considering growth and condition indices to assess fish health status following oil spill. Their complementary analysis with sensitive molecular biomarkers as EROD could improve the determination of oil spill impact on fish populations.

Key words : EROD, Growth, GST, Lipid index, PAHs, RNA:DNA

1. Introduction

Over ten events of oil tankers with important wastes have occurred in Europe since 1967. The Atlantic coast is in one of the main routes of the oil tankers and is therefore one of the most affected zone. The more important tanker accidents was the *Amoco Cadiz* in 1978, the *Exxon Valdez* in 1989, the *Erika* in 1999 and the *Prestige* in 2002. Despite the fact that oil spills are not the major contributors to oil released in the coastal environment (Kennish, 1992), estimates for a number of large spills suggest that 1 to 13% of the spilled oil can enter subtidal regions (Lee and Page, 1997). Assessment of the environmental impact of petroleum compounds on the marine environment is complicated because these products consist of complex mixtures of organic compounds. Moreover, after release into the marine environment, oil will gradually weather due to natural physical and chemical processes such as evaporation, dispersion, dissolution and chemical modification (Fingas and Hollebone, 2003). Risk assessments based solely on chemical analyses do not suffice to evaluate the complex toxic effects on marine species. The evaluation of oil spill impacts on marine organisms involves not only using adapted biomonitoring tools to indicate the magnitude of the exposure, but also understanding the potential long term impact on populations.

Crude oils are composed of many thousands complex gaseous, liquid and solid organic compounds of which hydrocarbons are the most abundant (Kennish, 1992). In particular, the presence of polycyclic aromatic hydrocarbons (PAHs) in crude oil is of concern, as they exhibit high chronic toxicity in the marine ecosystem (Achuba and Osakwe, 2003; Gonzalez-Doncel et al., 2008). As PAH compounds of such mixture could exhibit both antagonist and agonist effects, various magnitudes of deleterious effects can be observed according to the species and the concentrations occurring in marine environment (Billiard et al., 2006, Bado-Niles et al., 2009). Greater acute toxicity is generally associated with the lower molecular weight PAHs whereas some high molecular weight PAH form metabolites that can function as carcinogens (Gonzalez-Doncel et al., 2008).

PAHs are primarily metabolized, and thereby detoxified, by catalytic activities linked to the cytochrome P450 system (Sturve et al., 2006). The 7-ethoxyresorufin-O-deethylase (EROD) activity appears to be one of the most sensitive catalytic probe for determining the inductive responses of the cytrochrome P450 1A (Martinez-Gomez et al., 2006). This parameter was successfully used to monitor oil exposure related to Prestige oil spill (Martinez-Gomez et al., 2006). A dose-dependant induction of EROD was observed on Atlantic god, (*Gadus morhua*), exposed to a crude oil during 24 h (Abrahamson et al., 2008)

and 15 days (Sturve et al., 2006). Similar results were observed on juvenile salmons (*Salmo salar*) exposed to petroleum compounds after 4 and 8 days (Gagnon et Holdway, 2000). In parallel, Glutathione-S-transferases (GSTs) are the most important enzymes of the phase II biotransformation of xenobiotics that have been shown to respond to organic contaminants (Deviller et al., 2005; Castro et al., 2004). For example, Simonato et al. (2008) observed an increase in GST activity of juvenile neotropical fish (*Prochilodus lineatus*) exposed to the water-soluble fraction of diesel oil (WSD) and recommended GST activity as a sensitive biomarker for contamination by petroleum. PAH metabolization can also lead to the formation of reactive oxygen species (ROS) through the formation of redox labile metabolites. Antioxidant enzymes, such as catalase (CAT), belong to the cellular antioxidant system that counteracts the toxicity of ROS. Variations in CAT activity have been observed in fish exposed to organic compounds (Achuba and Osakwe, 2003; Damasio et al., 2007).

Considering the potential use of such molecular biomarkers in ecological risk assessment, it is important to know how the changes detected are relevant. Indeed, in spite of their rapid responsiveness and sensitivity to contaminant exposure, molecular biomarkers have questionable ecological relevance, as a result of being endpoints at a low level of biological organization (Castro, 2004). On the contrary, change in physiology and fitness seems to be a common response in marine organisms exposed to stressful pollutants (Alquezar et al., 2006; Faucher et al., 2008). Juvenile fish condition and health may be determined by a variety of indices, which are relevant at different time scales: biochemical indices (RNA:DNA ratio, lipid index), histological alterations, morphometric indices (Fulton's K) or growth indices. Mortality rates for fish are high during the first months of their lives, and only those few individuals surviving can reproduce and hence contribute to the recruitment success. Any difference in juvenile fish growth and condition in the first year of life can result in a large difference in the number of individuals entering the reproductive stage annually (Buckley et al., 1999). In this context, knowledge of the effects of contaminant exposure on the health of fish is essential in understanding the impact of pollution events, such as oil spills, on fish populations.

In the present study, deleterious effects of petroleum hydrocarbons were assessed in juvenile sea bass exposed to an Arabian light crude oil. Sea bass which is widely distributed along the European coast is an estuarine representative species and therefore liable to be exposed to oil spills. Moreover, it is a commercial species with a high economic importance in fish farming. The purpose of this study was twofold. First, short term effects of 48 h and 96 h crude oil exposure were assessed by the measurement of several biochemical biomarkers:

EROD, GST and CAT activities. In a second part, several growth and condition indices were measured on exposed-fish kept in seawater for 28 and 26 days to analyse the consequences of this short term exposure on fish health.

2. Materials and methods

This experiment was conducted in accordance with the European Commission recommendation 2007/526/EC on revised guidelines for the accommodation and care of animals used for experimental and other scientific purposes. CEDRE (Centre de Documentation de Recherche et d'Expérimentations sur les pollutions accidentelles des eaux, France) is authorized to conduct experimentation on animals in its capacity as a certified establishment; according to the administrative order no. 2006-0429 dated 9 May 2006.

2.1. Chemicals

The Arabian Light crude oil, the Brut Arabian Light 110 (BAL 110), used for this study, was topped for 24 h at 110°C to remove the most volatile components. This was done in order to simulate the natural behaviour of the oil after it is released at sea and before it reaches coastal zones. BAL 110, similar to oil spilled by the *Amoco Cadiz* in 1978, possesses the following physicochemical characteristics: 0.860 of density at 20°C, 60 mPa s of viscosity at 15°C, 12% polar compounds, 34% aromatic hydrocarbons and 54% saturated hydrocarbons.

2.2. Experimental procedure

The experiment was realised in Mars-April 2009. One hundred and sixty juvenile sea bass (*Dicentrarchus labrax*), (Weight 4.87 ± 0.83 g; Age: five months) were obtained from a hatchery (Aquanord in France). The fish were acclimatised in a clean tank supplied with an open seawater circuit for one week. The photoperiod was set at a 10 h light and 14 h dark cycle. During the acclimation, the water temperature was 13 ± 0.5 °C and the fish were fed with a commercial fish food twice a day. The daily feeding amount was maintained at approximately 2% of the total fish weight. Before the beginning of the experiment each fish was anaesthetised in a 320 $\mu l.L^{-1}$ 2−phenoxyethanol solution, individually marked (Visual

Implant Tag, 1.2 mm x 2.7 mm, Northwest Marine Technology), weighed (near to 0.01 g) and measured for total length (near to 0.1 mm).

The experimental system was devised to continuously mix the oil compounds with seawater. At the beginning of the experiment, the petroleum mixture was introduced in the system and mechanically dispersed using a funnel (at the surface of a 300-L seawater tank), which was linked to a Johnson L450 water pump (at the bottom of the tank) (Milinkovitch et al., 2011). The system was a static water system and exposure studies were conducted at 15 ± 0.5 ◦C. Physicochemical parameters (temperature, salinity, oxygen and pH) were measured daily. The mechanically dispersed oil exposure was made by pouring 20 g of petroleum into the funnel. For the control condition (Ctrl), the same experimental system was used without oil addition. Each condition (Ctrl and oil exposure) was done in duplicate, thus four tanks were used.

Crude oil was added into the system one day before the fish placement. The beginning of fish exposure was considered after this time period (t_0) where forty juvenile sea bass were randomly distributed in each tank. Ten fish per tank were sampled following 48-h (t_{48}) and 96-h (t_{96}) of exposure. They were anaesthetised (2−phenoxyethanol), their livers were sampled and frozen in liquid nitrogen, and their gills were preserved in a formaldehyde solution (10 %). The remainders of the fish were stored at -20°C. A t_{48} and t_{96}, ten other fish per tank were transferred to clean seawater for 26 and 28 days respectively. Conditions were similar to those occurring in acclimation. After this growth period, the fish were identified, weighed and measured for total length. Their livers and gills were sampled as previously. Their muscles were stored at -20°C and their otoliths (sagittae) were extracted and preserved in ethanol (95%). For analysis of PAHs in fish following the 48 h and 96 h exposure, 15 fish per treatment were individually put in polypropylene bag and stored at -20°C.

2.3. Chemical analysis

2.3.1. Total Petroleum Hydrocarbons (TPH) concentrations in seawater

The TPH concentrations, which are the sum of dissolved hydrocarbon concentrations and the amount of oil droplets, were measured daily for both exposure conditions during the 96 h-exposure, using the mean of two replicates for each time. No measurement was realised during the 24 h preceding the fish placement. The TPHs in the seawater samples were extracted with 10 mL of pestipur-quality dichloromethane (99.8 % pure solvent, Carlo Erba Reactifs, SDS). After the separation of the organic and aqueous phases, the water was

extracted two additional times with the same volume of dichloromethane (2×10 mL). The combined extracts were dried on anhydrous sulphate and then analyzed using a UV spectrophotometer (UV–vis spectrophometer, Unicam) at 390 nm, as described by Fusey and Oudot (1976).

2.3.2. PAH concentrations in seawater

Seawater concentrations of 19 PAHs (alkylated and parents) were assessed daily during the 96h-exposure: the 16 US-EPA (1996) PAHs and 3 sulfur containing heterocyclic compounds (benzothiophene, biphenyl and dibenzothiophene). No measurement was realised during the 24 h preceding the fish placement. After sampling, preduterated internal standards (CUS-7249, Ultra Scientific, Analytical solutions) were added to the samples and a 24 h settling phase was undertaken to separate oil droplets and particulate matter from the seawater. Then, PAHs were extracted from the seawater using the stir bar sorptive extraction technique (SBSE—stir bar coated with PDMS, Gerstel), and analyzed using thermal desorption coupled to capillary gas chromatography–mass spectrometry (GC–MS). The GC was a HP7890 series II (Hewlett Packard, Palo Alto, CA, USA) coupled with a HP5979 mass selective detector (MSD, Electronic Impact: 70 eV, voltage: 2000 V). PAHs were quantified relative to the perdeuterated internal standards introduced at the beginning of the sample preparation procedure and according to published procedures (Roy et al., 2005).

2.3.3. PAH concentrations in fish tissues

The levels of 19 PAHs (parents) were determined in the fish sampled after both exposure times with a GC-MS-MS Agilent Technologies 7890A, using the procedure of Baumard et al. (1997) with some modifications. Five pools of muscle from three fish were analyzed per treatment. A volume of 10 µl of perdeuterated internal standards (CUS-7249, Ultra Scientific, Analytical solutions) was added to about 3 g of fish pool samples, and the obtained mixtures were digested for 4 h under reflux in 50 ml of an ethanolic solution of potassium hydroxide (2 M, Fisher Chemicals). After cooling, settling and addition of 20 ml of deionised water, the digest was extracted in a 250 ml funnel twice with 20 ml of pentane (Carlo Erba Reactifs, SDS). The extract was evaporated with a Turbo Vap 500 concentrator (Zyman, Hopkinton, MA, USA, at 880 mbar and 50 °C) to obtain 1 ml of concentrated extract. The purification of the extract was performed by its transfer to a silica column (5 g of silica). Hydrocarbons were eluted with 50 ml of pentane : dichloromethane (80 : 20, v:v, SDS) and concentrated to 200 µl by means of a TurboVap 500 concentrator (Zyman, 880 mbar, 50°C). Aromatic compounds

were analysed by GC–MS-MS and PAHs were quantified relative to the perdeuterated internal standards introduced at the beginning of the sample preparation procedure.

The same protocol was used to measure the PAH concentrations in fish sampled following the 28 and 26 days period in clean seawater. However, only two pools of three fish were analysed per treatment.

2.4. Biological analysis

Biochemical hepatic biomarkers and histological analysis on the gills were performed on the fish sampled after the two exposure times (t_{48} and t_{96}) and on the fish transferred to the clean seawater after their contamination for 28 and 26 days, respectively. The specific growth rates, the otolith recent growth index, the RNA:DNA ratio, the TAG:ST ratio and the morphometric index, were only measured on the sea bass sampled after their period in the clean seawater.

2.4.1. Molecular biomarkers analysis

The livers were homogenized in an ice-cold phosphate buffer (0.1 M, pH = 7.8) with 20% glycerol and 0.2 mM phenylmethylsulfonyl fluoride as a serine protease inhibitor with the tissue homogenizer Precellys 24 (Bertin Technologies, France). The homogenates were then centrifuged at 10,000 g at 4 °C, for 15 min and the post-mitochondrial fractions were used for biochemical assays. The total protein concentrations were determined using the method of Bradford (1976) with bovine serum albumin (Sigma-Aldrich Chemicals, France) use as a standard.

Ethoxyresorufin-O-deethylase activity (EROD) was determined following the hydroxylation of 7-ethoxyresorufin by the method of Flammarion et al. (1998). The reaction mixture consisted of a phosphate buffer (0.1 M, pH = 6.5), 7-ethoxyresorufin (8 µM) and NADPH (0.5 mM). The change in fluorescence was recorded (excitation wavelength 530 nm, emission wavelength 585 nm) and the enzyme activity was expressed as $pmol.min^{-1}.mg^{-1}$ protein with resorufin use as a standard.

Glutathione S-transferase activity (GST) was determined following the conjugation of reduced glutathione with CDNB by the method of Habig et al. (1974). The reaction mixture consisted of a phosphate buffer (0.1 M, pH = 6.5), reduced glutathione (1 mM) and CDNB (1 mM). The change in absorbance was recorded at 340 nm and the enzyme activity was calculated as $\mu mol.min^{-1}.mg^{-1}$ protein with GST use as a standard.

Catalase activity (CAT) was determined by the method of Babo and Vasseur (1992). Briefly, the assay mixture consisted of a phosphate buffer (100 mM pH 6.5) and H_2O_2 (28 mM). The change in absorbance was recorded at 240 nm. CAT activity was calculated in terms of µmol.min^{-1}.mg^{-1} protein with bovine erythrocyte catalase use as a standard.

2.4.2. Histological analysis

Sea bass were analyzed to determine the histopathological damages in gills. After dehydration in graded concentrations of ethanol, the samples were embedded in a paraffin wax. Histological sections were stained with Hemalun Eosine Safran. These sections were then examined by light microscopy and photographed.

2.4.3. Growth indices

Sea bass specific growth rates in weight (% per day) were estimated as:

$$GW = 100(\ln W_2 - \ln W_1)/(t_2 - t_1),$$

where (W_1) and (W_2) are fish total body weight at times (t_1) (beginning of the experiment) and t_2 (time of collection). Similarly, the specific growth rate in length was estimated as:

$$GL = 100(\ln L_2 - \ln L_1)/(t_2 - t_1),$$

where (L_1) and (L_2) are fish total length at times (t_1) and (t_2) respectively.

The recent growth index (RG) was determined by measuring the width of the peripheral daily increments of the otoliths. As there was a significant relationship between the otolith sagittal diameter and fish length (diameter = 1.08 + 0.028 (TL), $R^2 = 0.67$, $p < 0.001$), daily otolith width increments from the previous 5 days before the end of the experiment were used as an indicator of recent growth (mean distance between the margin of the otolith back to the 5th ring). The sagittae were cleaned and photographed to determinate the diameter, perimeter and area of each otolith. Then, they were mounted on a glass slide in thermoplastic cement (Crystal Bond). Sections of sagittae were obtained by polishing them on both sides with grinding paper of decreasing grit sizes (5 to 0.1 µm) until increments at the outer edge were visible. Otoliths were etched for 30 sec with 0.1 M EDTA and analysed under transmitted light, using a video system fitted to a compound microscope. All the measurements were done along the same axis (anteroposterior) using an Image Analysis System (TNPC, 5.0, NEOSIS).

The increment measurement was repeated three times by the same experimenter at different intervals of time and the mean were calculated.

2.4.4. Condition Indices

Three condition indices were estimated: RNA:DNA ratio and TAG:ST ratio as indicators of nutritional status and Fulton's K condition index as an indicator of the fish general well being. This latter morphometric index assumes that heavier fish for a given length are in better condition. We calculated Fulton's K condition index with the formula: $K = 100(W/L^3)$, where (W) is the body mass (mg) and (L) is the total length (mm).

Nucleic acid quantification and subsequent RNA:DNA ratios have been used in numerous studies as indices for nutritional condition and growth assessment in larvae and juvenile fish (Buckley, 1984; Gwack and Tanaka, 2001). This biochemical index reflects variations in growth−related protein synthesis, since the quantity of ribonucleic acid (RNA) varies with the rate of protein synthesis, while the amount of desoxyribonucleic acid (DNA) per cell is species−constant in somatic tissue. The procedure used to determine RNA and DNA concentrations in each of the individual fish was based on the Clemmesen method (1988). Nucleic acids were measured on muscle fragments (0.05 g) by homogenizing the sample in an ice−cold Tris–EDTA buffer (0.05 M Tris, 0.1 M NaCl, 0.01 M EDTA, pH 8.0) using an Ultraturrax and subsequently transferring this to a mixture of a Tris–EDTA buffer, proteinase−K (pro−K) and sodium dodecyl sulfate (SDS). Nucleic acids were extracted by a purification step involving phenol−chloroformisoamylalcohol (Amara et al., 2009). The quantity of RNA and DNA was determined by the fluorescence−photometric technique using a specific nucleic acid fluorescent dye−ethidium bromide (Sigma−Aldrich Chemicals, France). The fluorescence due to the total RNA was calculated as the difference between the total fluorescence (RNA and DNA) and the fluorescence after RNAase treatment, which is assumedly due to the presence of DNA only. Salmon sperm DNA (Sigma−Aldrich Chemicals, France) and yeast type III RNA (Sigma−Aldrich Chemicals, France) were used as standards. RNA and DNA contents were both expressed as milligrams per µL.

The third condition index was a lipid storage index based on the ratio of the quantity of triacylglycerols (TAG; reserve lipids) to the quantity of sterols (ST; structural lipids) in the fish. The TAG content is dependent on the nutritional state of the fish as they are a main reserve of energy in teleosts and the first components to be mobilised during periods of stress, while sterol contents remain essentially unchanged during starvation (Galois et al., 1990). The amount of total lipids in each individual was measured on a sample of lyophilised muscle

(0.07 g). Lipid extraction was conducted using the method of Bligh and Dyer (1959) slightly modified as described by Meziane and Tsuchiya (2002). Lipids were extracted according to a mixture of water:chloroform:methanol (1:1:1, v/v/v). TAGs and sterols were separated from other lipids by performing thin layer chromatography (TLC).

2.5. Statistical analysis

All statistics were performed with XLSTAT 2007. Similarity between replicate tanks were analysed by a two-way ANOVA. As no difference was observed for all measured biomarkers, data from both treatment replicates were pooled (GW: $p = 0.310$; GL: $p = 0.698$, RG: $p = 0.681$, K: $p = 0.806$, TAG:ST: $p = 0.128$, RNA:DNA: $p = 0.976$, EROD: $p = 0.806$, GST, $p = 0.948$, CAT: $p = 0.834$). The differences in biological parameters between the control fish and the petroleum exposed fish at t_{48h} and t_{96h} were analysed with Student t tests. If any biological data did not comply with the parametric assumption of normality and homogeneity of variance after various transformation techniques were tested, the Mann−Whitney U test was used. This non−parametric test was also used to analyse differences in PAH concentrations in the fish.

3. Results

No prevalent mortality was observed during the exposure and the growth period; only five of the 160 (3 %) fish died. In fact, only one control fish died during the 96 h exposure, and one control fish and three oil-exposed fish died during the depuration period.

3.1. Hydrocarbon concentrations in seawater

Seawater total petroleum hydrocarbons (TPH) concentrations during the 96 h exposure of sea bass to petroleum are presented in Fig. 1. No TPH was detected in the control. During the first 48 h of fish exposure, TPH concentrations decreased from 18.6 ± 3.6 mg.L^{-1} to 9.60 ± 2.86 mg.L^{-1}. After $t_{48,}$ TPH concentrations increased to 19.8 ± 0.1 mg.L^{-1} and decreased again to 14.5 ± 2.6 mg.L^{-1} at t_{96}.

Seawater concentrations of the 19 PAHs (alkylated and parents) are presented in Table 1. At the beginning of the exposure, the total PAH concentration detected in the control was 0.43

± 0.23 µg.L^{-1} , whereas concentrations in oil condition was 48.7 ± 15.7 µg.L^{-1}. It appears that two- or three-ring PAH compounds were dominant when compared to heavier PAHs (≥ four rings). Similarly to TPH concentrations, a decrease in the total PAH concentration of 37 % was observed during the first 48 h. A reduction of 64 % in the total PAHs was observed between 48 h and 96 h.

3.2. PAH concentrations in fish

The PAH concentrations measured in the sea bass at the two time intervals of exposure are presented in Table 2. A significant increase in the total PAH concentrations in fish tissues was observed between the control (t_{48} : 500 ± 140 ng.g^{-1}) and the petroleum-exposed sea bass (t_{48} : 5 720 ± 1 410 ng.g^{-1}) after 48 h of exposure ($p = 0.016$). A significant decrease in PAH concentrations in fish was then observed following 96 h of exposure (t_{96} : 2 290 ± 470 ng.g^{-1}) compared to 48 h ($p = 0.010$). Similarly to the PAHs analysed in seawater, light PAHs were dominant in sea bass tissues. Indeed, significant differences between treatments were only observed for 2-3-ring PAHs. No 6-ring PAHs were detected in any fish. Following the 28 and 26 days in clean seawater, no difference in PAH concentrations between control (t_{48} : 0.33 ± 0.08 µg.g^{-1} ; t_{96} : 0.70 ± 0.15 µg.g^{-1}) and oil exposed sea bass (t_{48} : 0.96 ± 0.89 µg.g^{-1} ; t_{96} : 0.50 ± 0.17 µg.g^{-1}) was observed.

3.3. Molecular biomarker responses

EROD activities detected in the control sea bass ranged from 0.01 to 0.02 pmol.min^{-1}.mg^{-1} protein. When exposed for 48 h to petroleum hydrocarbons, their activities increased significantly until 2.74 ± 0.14 pmol.min^{-1}.mg^{-1} protein ($p < 0.001$) (Table 3a). A significant higher induction was observed after the 96 h of oil exposure (6.84 ± 0.26 pmol.min^{-1}.mg^{-1} protein) ($p < 0.001$). No significant difference in GST activities was observed in the juvenile sea bass exposed to petroleum for 48 h ($p = 0.735$) whereas a significant increase was recorded following the 96 h of oil exposure (0.67 ± 0.02 µmol.min^{-1}.mg^{-1} protein) compared to control (0.26 ± 0.02 µmol.min^{-1}.mg^{-1} protein) ($p < 0.001$). CAT activity measured in control fish was 1.31 ± 0.06 µmol.min^{-1}.mg^{-1} protein and no significant variation was observed in the sea bass after 48 h ($p = 0.260$) and 96 h ($p = 0.677$) oil exposure.

After the growth period in clean seawater (28 days for t_{48} and 26 days for t_{96}), a significant induction of EROD activity was still observed in the sea bass exposed to petroleum for 48 h ($p < 0.001$) compared to control fish (Table 3b). A significant induction of EROD and GST activities was also still observed in the sea bass exposed to petroleum for 96 h ($p < 0.001$). However, these inductions of EROD and GST activities were significantly lower compared to those observed just after the contamination ($p < 0.001$).

3.4. Gills histology

The only gill cell alteration observed in any of the samples was telangectasia (= branchial vessel aneurysm) (Fig. 2). However, the rates of anomalies were equally observed in both control and treated fish. This alteration was moderately or severely observed for 15 % and 10 % of control fish and for 10 % and 0 % of oil exposed fish during 48 h and 96 h, respectively. No anomaly was observed in the fish transferred to clean seawater for 28 and 26 days.

3.5. Growth and condition indices

Specific growth rates and recent growth were individually measured on the tagged fish following the growth period in clean seawater (Fig. 3). When exposed for 48 h, the sea bass specific growth rate in weight and the recent growth showed no difference between the control fish and the petroleum exposed fish. On the contrary, the specific growth rate in length was significantly lower in the sea bass exposed to oil for 48 h (0.20 ± 0.03 %.days^{-1}) compared to the control fish (0.33 ± 0.02 %.days^{-1}, $p = 0.001$). After the 96 h exposure to crude oil, specific growth rates in weight (0.22 ± 0.10 %.days^{-1}, $p = 0.024$), in length (0.19 ± 0.02 %.days^{-1}, $p = 0.004$) and the recent growth (8.71 ± 0.43 µm, $p = 0.015$) were significantly lower compared to the control sea bass (GW : 0.50 ± 0.03 %.days^{-1}; GL : 0.27 ± 0.02 %.days^{-1}; RG : 10.1 ± 0.3 µm).

Several indices related to the condition, the growth and the nutritional status of the fish were estimated (Fig. 4). No difference in Fulton's K condition indices ($p = 0.806$), RNA:DNA ratios ($p = 0.973$) and lipid indices ($p = 0.955$) was observed between both controls of the two exposure times. Whereas no significant difference was observed in the Fulton's K condition index of the sea bass exposed to oil for 48 h ($p = 0.460$), a significant decrease of this index

was observed after 96 h of petroleum exposure (1.06 ± 0.03 mg.mm^{-3}, p = 0.018) compared to the control fish (1.17 ± 0.02 mg.mm^{-3}). For both exposure times, a significant decrease of the RNA:DNA ratio was observed in the fish exposed to oil (t_{48} : 2.95 ± 0.16, p = 0.002; t_{96} : 3.38 ± 0.13, p = 0.032) compared to the control fish (t_{48} : 4.03 ± 0.28; t_{96} : 4.19 ± 0.29). No difference in the lipid index, based on the quantity of TAG on ST, was observed in the sea bass exposed to oil for 48 h (1.25 ± 0.27, p = 0.512) and 96 h (1.11 ± 0.22, p = 0.205) compared to the control fish (t_{48} : 1.57 ± 0.33; t_{96} : 1.49 ± 0.31).

4. Discussion

4.1. Petroleum hydrocarbon and PAH concentrations

The juvenile sea bass were exposed to a TPH concentration corresponding to that encountered under some oil spill situations. For instance, Lunel (1995) monitored 1-100 mg.L^{-1} of total petroleum hydrocarbons in coastal waters around Shetland during the *Braer* oil spill in 1993. Similarly, seawater PAH concentrations measured at the beginning of the exposure were of the same order of magnitude as those described in the literature after different oil spills. Seawater PAH concentrations ranged up to 28.8 µg.L^{-1} after the Prestige oil spill in 2002 (Gonzalez et al., 2006; Bado-Nilles et al., 2009) and up to 49.7 µg.L^{-1} four days after the *North Cape* oil spill in 1996 (Reddy and Quinn, 2001).

No oil slick was observed in either tank of oil exposure condition due to the energy of the experimental system. However, during the exposure, some oil droplets adhered to the experimental system and caused the decrease in TPH concentration. An increase was observed after the 48 h of exposure, probably because of the first sampling of fish which have probably put in suspension some oil droplets. In field situation, the fate and behaviour of oil spills in the environment depends on a number of physicochemical factors, including evaporation, dissolution and interaction between oil and sediments (Wang et al., 1999). The combination of these processes, called ‘weathering’, reduces the concentrations of hydrocarbons in water. In particular, in the initial stages of an oil spill, one of the most important processes that produce changes in hydrocarbon concentrations is the evaporation of the more volatile components (Nordvik, 1995). In the present study, crude oil was topped at 110°C before introduction into the experimental system. It could be then suggested that evaporation of volatile compounds was nearly complete at the beginning of the fish exposure. The observed decrease of PAH concentrations in seawater could be associated with the fish

absorption via passive diffusion through the gills, intestine and skin, since PAHs accumulate in lipid-rich tissues (Varanasi, 1989). Indeed, the main compounds of the crude oil used in this study were found in fish muscles following the 48 and 96 h of exposure. An increase in PAH concentrations with the time of exposure was not observed. On the contrary, they tended to decrease after the 96 h exposure. PAHs are hydrophobic compounds that accumulate in fat and lipid structures such as cellular membranes. However, PAHs are also quickly metabolized through specific pathways (Budzinski et al. 2004). After the 96 h exposure, a part of the PAHs would have been already metabolized in those fish exposed to petroleum. No prevalent difference in PAH concentrations was observed between the sea bass petroleum exposed and the control fish after 28 and 26 days of depuration, probably because of the PAH excretions. The fact that PAHs were found in fish tissues after the contamination is evidence of fish exposure to petroleum, and suggests a potential risk to fish health due to the mutagenic and/or carcinogenic properties known of PAHs.

4.2. Molecular biomarker activities

Metabolic enzymatic activities provide a biologically significant consequence of exposure to contaminants (Gagnon and Holdway, 2000). In particular, EROD was successfully used to monitor oil exposure related to the *Erika* and *Prestige* oil spillages (ICES, 2001; Martinez-Gomez et al., 2006). In the present study, an increase in EROD activity was observed in the juvenile sea bass after 48 h of exposure to petroleum. Among the different PAH compounds present in crude oil, several studies have shown that high molecular weight PAH, such as the four to six ring PAHs, are the more potent inducers of EROD activities (Gonzalez-Doncel et al., 2008). However, the light crude oil used in our study was mainly composed by low molecular weight PAHs, suggesting their ability to increase EROD activities. Sea bass EROD activities doubled after exposure of 96 h. Gagnon and Holdway (2000) reported also a time-dependant of EROD activites detected in juvenile Atlantic salmon (*Salmo salar*) exposed to petroleum compounds for 8 days. Since PAH concentrations in both seawater and fish muscles decreased at 96 h experiment, highest level of EROD could be due to large amount of metabolites produced and their ability to induce biotransformation system in fish (Aas and Klungsaryr, 1998)

GST is a well known biomarker involved in the conjugation of electrophilic compounds (or phase I metabolites) with glutathione, and is one of the main enzymes involved in xenobiotic phase II metabolism (Van der Oost et al., 2003). In the present study, GST

induction was observed in those sea bass exposed to crude oil, but an increase was only observed after four days of exposure. The 48 h exposure to crude oil appears not to have been sufficient to induce this enzyme. Similar differences in biomarker sensitivity between EROD (phase I) and GST (phase II) have been found previously for polar cod (*Boreogadus saida*) exposed to the WSF of crude oil (Nahrgang et al., 2010). In the same way, EROD activity has been found to be the most discriminating in a monitoring study of two demersal fish species, the four-spot megrim (*Lepidorhombus boscii)* and the dragonet (*Callionymus lyra*), following the *Prestige* oil spill (Martinez-Gomez et al., 2009).

The EROD and GST inductions observed after 96 h of exposure, and after 48 h for EROD, were still observed after the depuration period in clean seawater with lower values. This result could be related to the biochemical reactivity of the fish. For example, in Gagnon and Holdway (2000), the EROD activity measured in Atlantic salmon (*Salmo salar*) exposed to crude oil WAF recovered to control levels in only 2 to 6 days after transferred to clean seawater. For other species, EROD takes longer to be induced and longer to return to background levels as observed in Nahrgang et al. (2010) in which EROD and GST were still induced following two weeks of depuration of Polar cod (*Boreogadus saida*) dietary exposed to crude oil.

The organ most commonly involved in the biotransformation of exogen compounds is the liver, because of its function, position and blood supply (Van der Oost et al., 2003). In particular, the activities of the antioxidant enzymes, which defend the organisms against ROS, are critically important in the detoxification of radicals to non-reactive molecules. Dose-dependent increases in CAT activity in the liver and other organs were found in African catfish (*Clarias gariepinu*s) exposed for 196 h to crude oil (Achuba and Osakwe, 2003). On the contrary, barbel (*Barbus meridionalis*) collected in the river Fluvia (NE Catalunya, Spain) 5 months after an oil spillage, showed decreased levels of CAT activity (Damasio et al., 2007). In the present study, no difference in CAT activity was observed between the petroleum exposed sea bass and the control group. Juveniles of curimba (*Prochilodus lineatus*), exposed to the water-soluble fraction of diesel oil (WSD) for 24 and 96 h showed no difference in CAT activity (Simonato et al., 2008). Antioxidant enzymes are generally less responsive to pollutants than phase I and II biotransformation parameters, and the relationships between response and contaminant exposure are still less well established. However, the fact that hepatic CAT activity was not induced does not exclude the possibility that there was ROS formation after exposure to oil, because other enzymes can metabolize hydroperoxides (Van der Oost et al., 2003).

These results are part of a current project: DISCOBIOL project (DISpersant and response techniques for COastal areas; BIOLogical assessment and contributions to the Regulation). Primarily, this project intends to assess the toxicity of mechanically and chemically dispersed oil on several species living in nearshore areas. Consequently, this protocol of oil exposure was applied to *Crassostera gigas*, *Mytilus edulis*, *Scophtalmus maximus* and *Liza. aurata*. A decrease of CAT activities was observed in the Pacific oyster, *Crassostrea gigas* (Luna-acosta et al., 2011) and no difference in EROD, GST or CAT activities was observed in juvenile golden grey mullet, *Liza aurata*, following 48 h of exposure (Milinkovitch et al., 2011). These differences between golden grey mullet and sea bass could be associated with species-specific responses, or attributed to size of the fish; since the mullet was 140 mm long (Milinkovitch et al., 2011). Earlier life stage sea bass (74 mm long) would have been more sensitive to oil exposure.

A variety of parameters in fish have been associated with the induction of EROD and GST activities, including reproductive effects (e.g. reduced serum steroid levels), increased liver somatic index and mortality (Whyte et al., 2000). Moreover, in some cases, biotransformation generates active intermediates, which are able to interact with vital cellular macromolecules, such as DNA, thereby causing mutagenic and carcinogenic effects (Goldstein and Falleto, 1993). In the present study, as EROD induction was observed after 48 h of exposure and GST induction after 96 h, effects on fish health could be suggested. However, as a direct link between biotransformation system induction and detrimental effects in fish has not been clearly established, their activity is best viewed as an indicator of contaminant exposure rather than an effect.

4.3. Gill histology

The exposure of fish to chemical contaminants induces a number of modifications in different organs particularly gills, kidney and liver, which histological examination represent a useful tool to assess the effects of xenobiotics (Giari et al., 2007). Haensly et al. (1982) reported histopathological abnormalities in gills of plaice (*Pleuronectes platessa*) that were exposed to the Amoco Cadiz oil spill in an isolated inlet off the coast of France. In our study, the only alteration evident in samples was telangectasia, but the anomaly was observed in both control and oil exposed fish, maybe due to their manipulation. This absence of difference might be related to the period of exposure. For example, some lesions such as hepatocellular neoplasms might take two or more years to develop in fish species (Schiewe et al., 1991).

4.4. Growth and condition indices

Since molecular biomarkers have shown missing ecological relevance, their induction are hardily predictable of potential damages in fish development and survival. On contrary, it is likely that changes in individual health, manifest themselves at higher levels of ecological organisation, leading to reduced fish recruitment success, abundance and production. For instance, Lancaster et al. (1998) analysed the growth and condition of sea bass following the Sea Empress oil spill, and suggested a detrimental effect on the local abundance of juvenile bass following the event.

Several studies have shown that crude oil and its components inhibit the growth of fish in a number of species. This is especially the case at earlier life stages, such as larvae and juveniles stages, when the fish are exposed to WSF of crude oil (Al-Yakoob et al., 1996), oil-laden sediments (Moles and Norcross, 1998), or as a result of ingestion of oil-contaminated food (Saborido-Rey et al., 2007). Since somatic growth requires the fish to be exposed a long time before significant differences can be detected (National Research Council, 2002), most growth studies have been done under chronic exposure to petroleum. However, little is known about the long term consequences of short acute exposure to crude oil; that is why in the present study, a growth period in clean seawater was respected after acute exposure. The sea bass exposed to the crude oil presented a significant decrease in somatic growth in length after 48 h of exposure, but a significant difference in specific growth rate in weight was only observed after 96 h of exposure. Variations on the scale of a few days, over the month of depuration, can be detected by analysis of otolith growth and increment widths. Otolith growth reflects both fish growth and metabolism, and therefore is a sensitive indicator of the physiological state of an individual (Morales-Nin et al., 2007). A difference in sea bass recent growth was only observed after 96 h of exposure compared to the control group. Growth reductions in this study could be attributed to a combination of different actions of petroleum compounds on fish like an increase in metabolism due to detoxification. Growth impairment during the juvenile stage is likely to be critical to survival and recruitment. Fish experience their most rapid growth during the juvenile phase (Smith et al., 1995), and any reduction during juvenile growth would prolong the duration of this life period. If oil inhibits sea bass growth, these fish would be more susceptible to predation and might compete less successfully for food than larger fish (He, 2010)

The combined use of biochemical and morphometric condition indices could be of great interest to analyse the energetic condition of fish following exposure at different time responses. Nucleic acids have provided useful tools for assessing the growth and nutritional condition of both larval and juvenile fish (Kuropat et al., 2002). In the present study, this index was correlated ($R = 0.334$, $p = 0.022$) with the specific growth rate in length and presented the same evolution with a significant decrease in the sea bass RNA:DNA ratio, 28 and 26 days after the 48 and 96 h exposure time to petroleum. Although, this index is considered to be a short-term indicator (Gilliers et al., 2006), its reduction observed 28 days following the 48 h of petroleum exposure suggested its sensitivity. On the other hand, the morphometric index based on length-weight data revealed a reduction in sea bass condition only following the 96 h exposure. The Fulton's K index is considered as a long-term indicator of individual's general well-being on the scale of weeks or months (Suthers, 2000). It was successfully used to show the negative effect of 24 h of fuel exposure on sole (*Solea solea*) transferred to clean seawater for 2 months (Claireaux et al., 2004), and in sole caught in sites exposed to the *Erika* oil spill (Gilliers et al., 2006). The decrease in the specific growth rate in length and the RNA:DNA ratios after 48 h exposure suggested the toxicity of petroleum and its deleterious effects on fish.

The use of lipid storage indices such as triacylglycerol on sterol ratio (TAG:ST) has been considered as relevant to integrate the nutritional status of fish (Suthers et al., 1992). TAGs are the major energy storage form in fish and have important ecophysiological relevance as indicators of growth potential and survival (Sogard and Olla, 2000). Xenobiotic detoxification involves energetic mechanisms (Alquezar et al., 2006) and it has been shown that fish exposed to crude oil have lower feeding activities (Moles and Norcross, 1998). Consequently a decrease in the TAG:ST ratio could be observed in response to oil contamination. Indeed, in a mesocosm study, Claireaux et al. (2004) showed that the value of the TAG:ST ratio of juvenile sole exposed for 24 h to PAHs diminished by 75% after a 3 months period in clean seawater relative to control fish. In the present study, fish were fed *ad libitum* with commercial pellets to favour growth performances, which have probably minimized any potential nutritional deficits.

4.5. Conclusions

In the present study, juvenile sea bass responses to 48 and 96 h of oil exposure were assessed at the sub-cellular level using some molecular biomarkers, and at the physiological

level using some growth and condition indices. The metabolic cost associated to PAH absorption in fish was showed by the rapid EROD induction as soon as 48 h and GST induction after 96 h. Effects of this short term exposure on fish health were then demonstrated by a decrease of fish growth during the month following the 48 h exposure accompanied with a decrease of fish condition index after the 96 h. This study show the complementarities of molecular and physiological biomarkers to assess crude oil exposure effects. This approach could be applied to survey oil spill or other oil rejects consequences. Indeed, the measurements of molecular biomarkers such as EROD, could give information on fish petroleum hydrocarbon exposure, while physiological biomarkers reflect fish health status.

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Figure legends

Fig 1. Total petroleum hydrocarbons (TPH) concentrations ($mg.L^{-1}$) in seawater during the 96 hour exposure of sea bass to petroleum. Values are expressed as mean of both experimental replicates (mean ± SE).

Fig.2. Histopathological analysis of sea bass gills: (A) normal gill filaments (B) section of secondary lamellae of petroleum-exposed sea bass showing severe telangectasia (arrows).

Fig. 3. Comparison of specific growth rate in length (GL), in weight (GW) and recent growth (RG) (mean ± SE), in control sea bass (CTRL) and petroleum-exposed sea bass (OIL) following 48 h and 96 h of exposure and following the 28 and 26 day period in clean seawater, respectively.
(*) represents the significant difference ($p < 0.05$) compared to respective control.

Fig. 4. Comparison of Fulton's K index, RNA:DNA ratio and TAG:ST ratio (mean ± SE), in control sea bass (CTRL) and petroleum-exposed sea bass (OIL) following 48 h and 96 h of exposure.
(*) represents the significant difference ($p < 0.05$) compared to respective control.

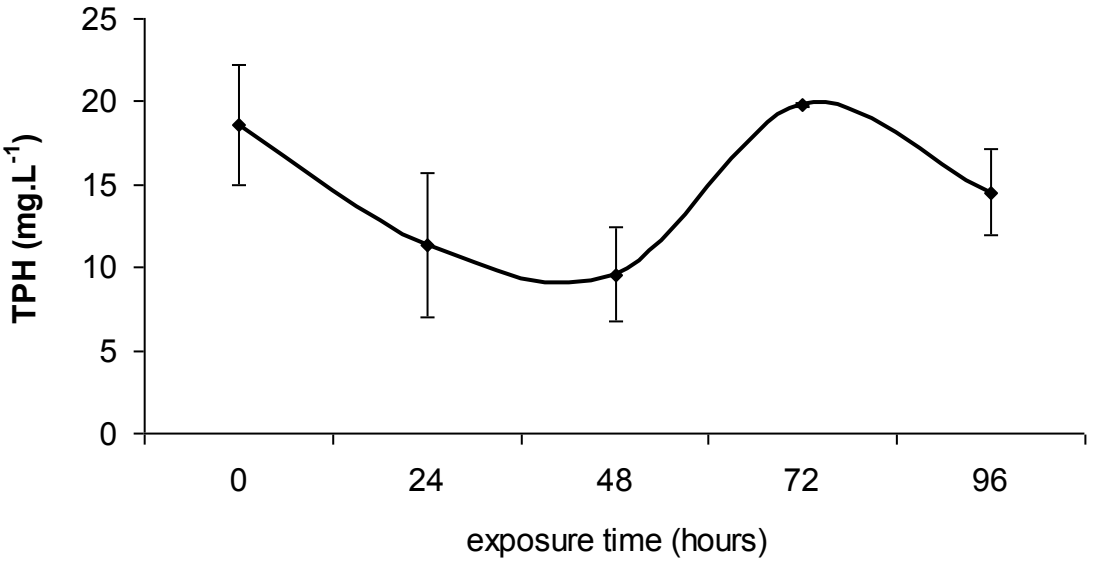

Fig. 1

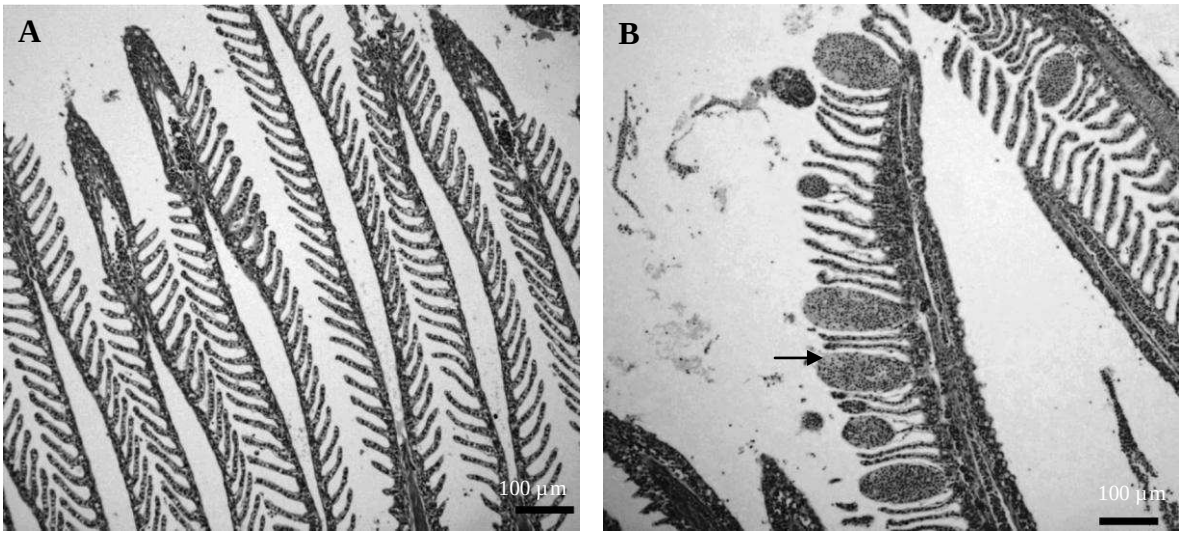

Fig. 2

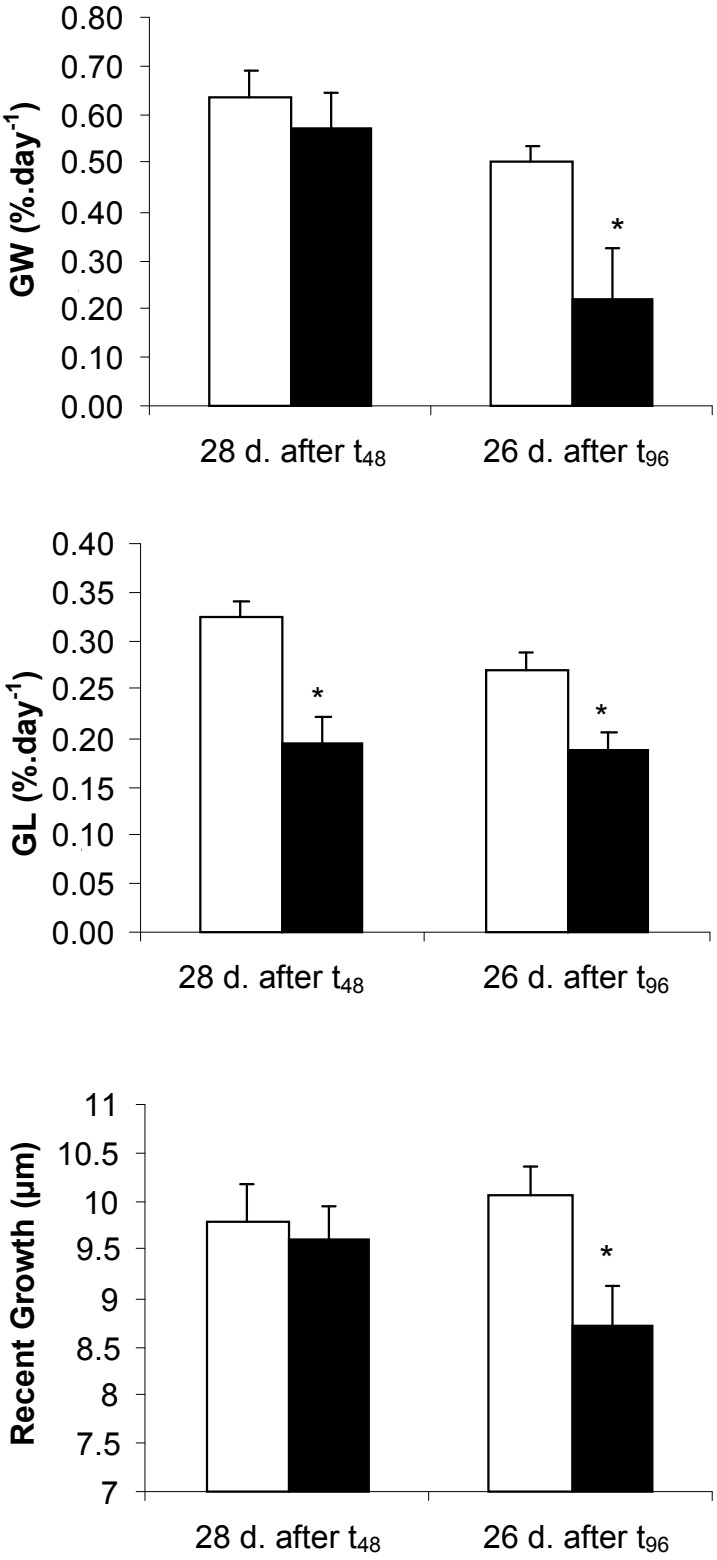
GW (%.day-1)
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
*
28 d. after t48
26 d. after t96
GL (%.day-1)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
*
*
28 d. after t48
26 d. after t96
Recent Growth (µm)
11
10.5
10
9.5
9
8.5
8
7.5
7
*
28 d. after t48
26 d. after t96

Fig. 3

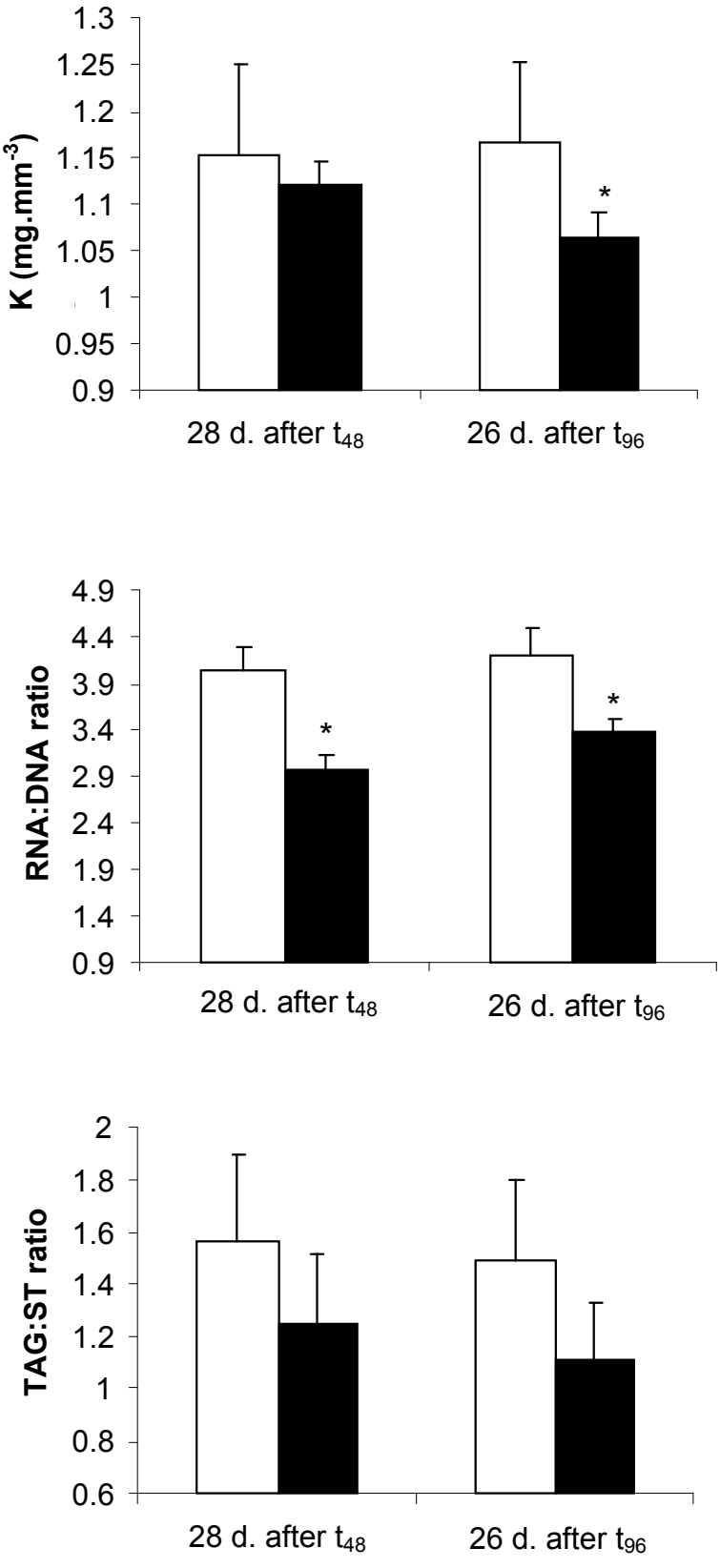
K (mg.mm-3)
1.3
1.25
1.2
1.15
1.1
1.05
1
0.95
0.9
*
28 d. after t48
26 d. after t96
RNA:DNA ratio
4.9
4.4
3.9
3.4
2.9
2.4
1.9
1.4
0.9
*
*
28 d. after t48
26 d. after t96
TAG:ST ratio
2
1.8
1.6
1.4
1.2
1
0.8
0.6
28 d. after t48
26 d. after t96

Fig. 4

Table legends

Table 1

Concentrations of 19 PAHs (alkylated and parents): the 16 US-EPA PAHs and 3 sulfur containing heterocyclic compounds ($ng.L^{-1}$) in seawater at the beginning (T= 0 h), at T = 48 h and at T = 96 h in CTRL (control) and OIL (petroleum exposure). Values are expressed as the mean of both experimental replicates (mean ± SE).

n.d.: not detected

Table 2

PAHs body burdens ($ng.g^{-1}$ dry wt) in muscles of control sea bass (CTRL) and petroleum-exposed sea bass (OIL) following 48 h and 96 h of exposure.

(*) represents the significant difference ($p < 0.05$) compared to the respective control

n.d. : not detected

Table 3

Differences in Ethoxyresorufin-O-deethylase (EROD, $pmol.min^{-1}.mg\ prot^{-1}$), Glutathione S-transferase (GST, $\mu mol.min^{-1}.mg\ prot^{-1}$) and Catalase (CAT, $\mu mol.min^{-1}.mg\ prot^{-1}$) measured on control sea bass gill (CTRL) and petroleum-exposed sea bass (OIL) following 48 h and 96 h of exposure (a.), and following 26 and 28 days of decontamination respectively (b.).

(*) represents the significant difference ($p < 0.05$) compared to the respective control

Table 1

PAH concentrations ($ng.L^{-1}$)	T = 0h		T = 48h		T = 96h	
	CTRL	OIL	CTRL	OIL	CTRL	OIL
2-ring						
Naphtalene	35.6 ± 8.41	911 ± 7.7	12.4 ± 3.78	591 ± 213	11.9 ± 1.74	323 ± 63.6
C1-Naphtalene	62.6 ± 3.20	5930 ± 1253	32.3 ± 9.35	1620 ± 737	31.6 ± 2.50	638 ± 179
C2-Naphtalene	43.5 ± 43.5	11106 ± 4037	47.9 ± 5.04	1706 ± 593	28.6 ± 3.99	668 ± 174
C3-Naphtalene	34.9 ± 24.8	7635 ± 2848	31.2 ± 1.79	969 ± 151	15.9 ± 6.14	770 ± 69.3
C4-Naphtalene	8.03 ± 5.68	1366 ± 443	9.51 ± 1.19	3891 ± 1578	4.74 ± 2.44	938 ± 9.69
Benzothiophene	5.17 ± 4.09	21.09 ± 3.58	n.d.	36.3 ± 20.3	0.58 ± 1.00	1.40 ± 1.40
C1-Benzothiophene	4.52 ± 1.95	381 ± 98	4.19 ± 0.28	572 ± 169	6.28 ± 0.87	180 ± 180
C2-Benzothiophene	40.4 ± 40.4	4915 ± 1528	61.8 ± 5.13	1879 ± 456	90.5 ± 5.43	574 ± 451
C3-Benzothiophene	20.0 ± 20	5925 ± 2219	20.8 ± 0.60	889 ± 252	24.5 ± 5.30	674 ± 98.7
C4-Benzothiophene	12.1 ± 10.7	2705 ± 1141	13.0 ± 0.66	3105 ± 1014	14.1 ± 1.54	989 ± 6.82
Biphenyl	7.26 ± 1.52	140 ± 34.1	105 ± 10.2	1072 ± 711	110 ± 18.5	297 ± 167
3-ring						
Acenaphtylene	1.50 ± 1.00	110 ± 38.0	1.09 ± 0.22	27.3 ± 13.4	0.53 ± 1.00	6.14 ± 3.64
Acenaphtene	2.91 ± 1.00	68.6 ± 14.7	2.84 ± 0.49	14.9 ± 3.67	1.90 ± 1.00	4.65 ± 2.02
Fluorene	8.21 ± 0.76	385 ± 97.1	4.25 ± 0.66	33.4 ± 15.5	2.91 ± 1.00	9.94 ± 5.88
C1-Fluorene	8.32 ± 1.55	473 ± 145	7.57 ± 1.75	126 ± 51.6	4.55 ± 1.56	38.8 ± 18.0
C2-Fluorene	5.50 ± 1.76	314 ± 82.8	7.20 ± 2.57	959 ± 323	3.49 ± 1.00	130 ± 20.5
C3-Fluorene	2.18 ± 2.18	162 ± 34.0	5.57 ± 2.08	1462 ± 788	3.72 ± 1.00	301 ± 100
Dibenzothiophene	35.1 ± 1.6	1565 ± 447	16.8 ± 0.40	159 ± 68.7	10.9 ± 1.42	69.4 ± 48.0
C1-Dibenzothiophene	15.6 ± 15.6	1872 ± 516	20.8 ± 0.75	507 ± 220	11.1 ± 0.57	84.6 ± 32.8
C2-Dibenzothiophene	7.18 ± 7.18	911 ± 201	13.0 ± 3.14	4747 ± 2083	10.3 ± 1.00	998 ± 126
C3-Dibenzothiophene	2.57 ± 2.57	242 ± 51.3	4.98 ± 1.15	3090 ± 1842	5.65 ± 1.00	1226 ± 364
Phenanthrene	18.7 ± 4.36	396 ± 112	6.53 ± 0.89	34.8 ± 22.80	3.16 ± 1.00	30.7 ± 25.6
Anthracene	13.8 ± 10.4	8.39 ± 0.79	0.76 ± 0.09	14.1 ± 4.06	n.d.	56.5 ± 52.3
C1-Phen/Anthr	8.91 ± 2.13	496 ± 135	8.11 ± 2.43	97.75 ± 48.3	4.58 ± 1.26	46.2 ± 16.3
C2-Phen/Anthr	4.39 ± 0.55	210 ± 49.4	6.17 ± 2.85	796 ± 346	6.34 ± 2.05	246 ± 49.6
C3-Phen/Anthr	0.82 ± 0.82	58.7 ± 11.6	2.17 ± 1.12	733 ± 429	2.40 ± 1.00	440 ± 140
4-ring						
Fluoranthene	1.49 ± 1.49	9.72 ± 2.91	1.20 ± 0.35	49.8 ± 28.0	1.44 ± 1.00	15.1 ± 8.71
Pyrene	3.59 ± 1.00	13.4 ± 3.11	2.26 ± 0.96	158 ± 107	3.51 ± 1.53	98.7 ± 45.9
C1-Fluor/Pyrene	0.68 ± 0.68	16.2 ± 2.43	1.63 ± 1.00	243 ± 157	3.80 ± 1.63	244 ± 96
C2-Fluor/Pyrene	0.59 ± 0.59	20.2 ± 2.54	n.d.	329 ± 221	2.27 ± 2.27	361 ± 109
C3-Fluor/Pyrene	n.d.	18.9 ± 2.17	n.d.	269 ± 168	10.3 ± 6.19	274 ± 78
Benzo[a]anthracene	1.03 ± 1.03	9.27 ± 2.32	n.d.	2.99 ± 0.05	n.d.	2.15 ± 0.69
Chrysene	1.22 ± 1.22	40.2 ± 12.1	1.09 ± 1.00	33.8 ± 7.22	1.63 ± 1.00	24.9 ± 10.4
C1-Chrysenes	n.d.	34.1 ± 8.17	1.23 ± 1.00	29.0 ± 8.67	1.88 ± 1.88	23.9 ± 10.9
C2-Chrysenes	3.42 ± 3.42	200 ± 25.8	16.8 ± 2.46	137 ± 24.8	33.0 ± 9.18	86.3 ± 46.8
C3-Chrysenes	1.86 ± 1.86	37.1 ± 5.45	7.50 ± 1.00	21.6 ± 7.68	10.9 ± 1.00	30.4 ± 11.9
5-ring						
Benzo[b+k]fluoranthene	1.03 ± 1.03	2.37 ± 1.00	1.49 ± 1.00	4.99 ± 1.00	1.19 ± 1.00	2.03 ± 1.00
Benzo[a]pyrene	1.24 ± 1.24	2.37 ± 1.00	1.09 ± 1.00	2.49 ± 1.00	1.66 ± 1.00	2.47 ± 0.62
Benzo[g.h.i]perylene	n.d.	2.37 ± 1.00	n.d.	1.50 ± 1.00	n.d.	0.46 ± 1.00
6-ring						
Indeno[1.2.3-cd]pyrene	n.d.	24.5 ± 10.0	n.d.	18.5 ± 7.00	n.d.	5.67 ± 1.00
Dibenzo[a.h]anthracene	n.d.	30.8 ± 15.0	n.d.	22.4 ± 10.00	n.d.	6.89 ± 1.00
total PAHs ($\mu g.L^{-1}$)	0.43 ± 0.23	48.7 ± 15.7	0.48 ± 0.68	30.4 ±12.9	0.48 ± 0.93	10.9 ± 2.8

Table 2

	T = 48h		T = 96h	
	CTRL	OIL	CTRL	OIL
Naphtalene	127 ± 28.3	600 ± 196*	208 ± 39.7	346 ± 72.4
Benzothiophene	0.32 ± 0.37	49.2 ± 30.6*	0.85 ± 0.85	12.8 ± 2.80*
Biphényl	29.2 ± 12	273 ± 60.0*	38.5 ± 8.28	127 ± 9.19*
Acenaphtylene	n.d.	233 ± 61.8*	n.d.	82.5±14.4*
Acenaphtene	11.1 ± 7.82	330 ± 178*	23 ± 3.33	85.4 ± 10.9*
Fluorene	55.7 ± 25.1	609 ± 164*	67.8 ± 7.65	211 ± 47.5*
Dibenzothiophene	158 ± 28.8	3210 ± 628*	178 ± 25.5	1174 ± 224*
Phenanthrene	99.1 ± 23.3	360 ± 56.3*	87 ± 22.6	165 ± 31.2
Anthracene	n.d.	7.11 ± 7.11	n.d.	37.5±25.2
Fluoranthene	4.01 ± 4.63	12.6 ± 7.99	5.49 ± 5.49	7.69 ± 7.15
Pyrene	4 ± 4.62	9.89 ± 9.89	4.92 ± 4.92	18.7 ± 17.2
Benzo[a]anthracene	4.95 ± 5.72	2.97 ± 2.97	1.03 ± 1.03	5.12 ± 3.36
Chrysene	8.45 ± 5.38	22.5 ± 4.67	8.72 ± 3.19	18.4 ± 1.71
Benzo[b+k]fluoranthene	n.d.	n.d.	0.07±0.07	1.71±1.71
Benzo[a]pyrene	1.49 ± 1.01	2.7 ± 0.99	2.76 ± 1.01	1.77 ± 0.72
Perylene	n.d.	n.d.	n.d.	n.d.
Indeno(1.2.3-cd)pyrene	n.d.	n.d.	n.d.	n.d.
Dibenzo(a.h)anthracene	n.d.	n.d.	n.d.	n.d.
total PAHs	503 ± 147	5721 ± 1408	626 ± 124	2294 ± 469

Table 3

	T = 48h		T = 96h	
	CTRL	OIL	CTRL	OIL
EROD	0.01 ± 0.01	2.74 ± 0.14*	0.02 ± 0.01	6.84 ± 0.26*
GST	0.29 ± 0.03	0.31 ± 0.02	0.26 ± 0.02	0.67 ± 0.02*
CAT	1.31 ± 0.06	1.45 ± 0.10	1.51 ± 0.10	1.57 ± 0.10

a.

	28 d. after t_{48}		26 d. after t_{96}	
	CTRL	OIL	CTRL	OIL
EROD	0.01 ± 0.01	0.42 ± 0.08*	0.01 ± 0.01	1.15 ± 0.11*
GST	0.29 ± 0.02	0.24 ± 0.03	0.26 ± 0.02	0.40 ± 0.03*
CAT	1.50 ± 0.09	1.50 ± 0.11	1.57 ± 0.11	1.44 ± 0.08

b.